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Multiple mechanisms regulate c-*myc* gene expression during normal T cell activation

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Quiescent normal human T cells express low levels of steady-state c-*myc* mRNA as a result of low constitutive promoter utilization, a block to transcriptional elongation within the gene, and rapid degradation of c-*myc* mRNA in the cytoplasm. Following the activation of the T cell receptor (TCR)/CD3 complex, quiescent T cells are induced to express c-*myc* mRNA. Two intracellular pathways, one involving protein kinase C activation and the other mediated by increased intracellular calcium concentration, are activated by TCR/CD3 receptor stimulation. These two pathways, which can be activated by phorbol myristate acetate (PMA) and ionomycin respectively, appear to play complementary roles in the transcriptional induction of c-*myc* gene expression by the antigen receptor complex. Ionomycin treatment of quiescent cells leads to enhanced c-*myc* expression primarily as a result of increased transcriptional initiation. In contrast, PMA contributes to c-*myc* expression, at least in part, by decreasing the block to transcriptional elongation present within the gene. Both the PMA- and ionomycin-mediated induction of c-*myc* expression can be independently enhanced by stabilization of c-*myc* mRNA in the cytoplasm. These observations demonstrate that multiple mechanisms co-operate to regulate c-*myc* gene expression during normal T cell activation.

Key words: c-*myc* gene/gene expression/T cell activation/T cell receptor/transcriptional regulation

Introduction

Expression of the c-*myc* proto-oncogene has been implicated in the growth regulation of a variety of both normal and neoplastic cell types (for review see Cole, 1986; Rabbitts, 1985). Quiescent cells express low levels of both c-*myc* mRNA and protein (Smeland *et al.*, 1987). Stimulation of quiescent cells to initiate proliferation leads to a rapid accumulation of c-*myc* mRNA within 2 h following stimulation (Kelly *et al.*, 1983). Thereafter, c-*myc* mRNA and protein continue to be expressed throughout the proliferative response (Hann *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985). Expression of c-*myc* protein appears to be required for cells to traverse the cell cycle. Quiescent T cells treated with anti-sense c-*myc* oligonucleotides fail to enter S phase when stimulated with mitogens (Heikkila *et al.*, 1987; Harel-Bellan, 1988).

Various molecular alterations in the expression of the c-*myc* gene have been associated with the deregulation of cell proliferation (for review see Klein and Klein, 1985, Marcu, 1987). Increased transcriptional initiation within the c-*myc* locus has been reported as both a result of avian leukosis virus (ALV) integration 5′ of the c-*myc* gene in bursal lymphomas (Hayward *et al.*, 1981; Parchl *et al.*, 1983) and as a result of translocations which lead to juxtaposition of the immunoglobulin heavy-chain gene enhancer and the c-*myc* transcription unit (Bernard *et al.*, 1983; Hayday *et al.*, 1984). Enhanced transcriptional elongation within the c-*myc* gene has been reported in Burkitt's lymphoma cell lines which have point mutations within the c-*myc* gene at the 3′ end of the first exon and 5′ end of the first intron (Cesarman *et al.*, 1987). Post-transcriptional alterations of c-*myc* gene expression have also been reported (Dani *et al.*, 1984). In some transformed lymphoid cell lines, stabilization of the normally labile c-*myc* mRNA leads to enhanced steady-state c-*myc* mRNA levels (Eick *et al.*, 1985; Hollis *et al.*, 1988).

While the above data suggest that increased transcriptional initiation, loss of transcriptional attenuation/pausing and stabilization of cytoplasmic c-*myc* mRNA can all enhance c-*myc* gene expression in transformed lymphoid cells, relatively little is known about the molecular mechanisms that regulate c-*myc* gene expression during normal T cell proliferation. In addition, little is known about how receptor–ligand interactions on the cell surface of quiescent cells contribute to the regulation of c-*myc* expression. In order to address these issues, we initiated studies to investigate the molecular mechanisms by which stimulation of the TCR/CD3 complex on normal peripheral blood human T cells leads to the induction of c-*myc* mRNA expression. Our results demonstrate that quiescent human peripheral blood T lymphocytes express virtually no c-*myc* mRNA. Activation of quiescent T cells by crosslinking of the TCR/CD3 receptor complex results in a rapid rise in c-*myc* mRNA levels. Peak levels of c-*myc* mRNA are reached within 6 h of stimulation and abundant c-*myc* mRNA is expressed throughout the proliferative response. The proliferative response induced by crosslinking of the TCR/CD3 receptor can be mimicked by the simultaneous activation of resting peripheral blood T cells with phorbol myristate acetate (PMA) to induce protein kinase C activation and with ionomycin to increase intracellular ionized calcium levels. Treatment of resting T cells with these two agents also leads to the induction of c-*myc* mRNA levels that are similar in quantity and kinetics to the pattern of c-*myc* expression induced by crosslinking of the TCR/CD3 receptor complex. Treatment of peripheral blood T cells with either PMA or ionomycin alone does not induce a proliferative response. However, each agent is capable of inducing a distinct pattern of c-*myc* gene expression.

Activation of c-*myc* gene expression following treatment of T cells with ionomycin peaks within 1 h of stimulation, does not require new protein synthesis, and is mediated

primarily by increased transcriptional initiation. In contrast, PMA treatment of quiescent T cells leads to a gradual increase in c-*myc* mRNA over 12–24 h in culture, requires new protein synthesis, and is mediated, at least in part, by elimination of a block to transcriptional elongation within the gene. Neither of these agents leads to alteration in the normal c-*myc* mRNA half-life of 15–20 min. Addition of cycloheximide to resting peripheral blood T cells or T cells pretreated with PMA and/or ionomycin leads to additional increases in c-*myc* mRNA levels by increasing the half-life of c-*myc* mRNA. Together these results suggest that multiple mechanisms regulate c-*myc* gene expression during the proliferative response of normal human T cells.

Results

Expression of c-myc is induced following crosslinking of the TCR/CD3 complex

Previous studies have shown that both human and murine B lymphocytes can be induced to express c-*myc* mRNA following crosslinking of their surface immunoglobulin receptors (Smeland *et al.*, 1985; Buckler *et al.*, 1988). Since the TCR/CD3 complex appears to be the T cell equivalent of the imunoglobulin receptor, we first sought to generalize a role for the antigen receptor in the induction of c-*myc* expression during the proliferative response of normal human T cells. In order to activate the TCR/CD3 complex in a polyclonal population of cells, we have made use of an anti-CD3 monoclonal antibody attached to a plastic surface (CD3sp) to allow for efficient crosslinking of the TCR/CD3 complex. T cell activation by this monoclonal antibody results in initiation of growth as measured by increases in volume and total cellular RNA in >90% of resting peripheral blood T lymphocytes (data not shown). In addition, 40–60% of this polyclonal population will proceed through at least one round of proliferation in response to antigen receptor crosslinking. In order to perform these studies we have utilized a highly purified population of resting human peripheral blood T lymphocytes (June *et al.*, 1987). This population has been rigorously depleted of accessory cells that might produce lymphokines involved in secondary activation events following TCR/CD3 stimulation. Thus, this population of T cells does not proliferate *in vitro* in response to stimulation with PHA, a mitogen known to require accessory cells for induction of proliferation (Table I). RNA was isolated at different time points following CD3sp stimulation of this resting T cell population and c-*myc* mRNA expression was analyzed by Northern blot analysis (Figure 1). Resting T cells expressed only low levels of c-*myc* mRNA. However, c-*myc* mRNA was induced within 1 h following CD3sp stimulation, reached a maximal level 6 h following stimulation, and was thereafter expressed throughout the proliferative response.

c-myc mRNA expression in resting T cells can be induced by both PMA and ionomycin

Previous studies have shown that antibodies to the TCR CD3 receptor complex initiate their intracellular effects by activating phospholipase C to release inositol-1,4,5 trisphosphate and diacylglycerol from the membrane (Weiss and Imboden, 1987). These intracellular secondary messengers in turn lead to a rapid rise in cytoplasmic free calcium and the membrane translocation of protein kinase

Table I. Proliferation of quiescent peripheral blood T cells can be induced by crosslinking of the TCR CD3 receptor complex or the combination of PMA + ionomycin[a]

	Stimulus[b]	Thymidine incorporation (c.p.m.)[c]	
Exp. 1	Medium	246 ±	75
	CD3sp	126 000 ±	15 000
	PHA	513 ±	112
	PMA	1 230 ±	330
	PMA + CD28 mAb 9.3	209 000 ±	14 000
Exp. 2	Medium	121 ±	6
	PMA	800 ±	380
	Ionomycin	105 ±	15
	PMA + ionomycin	29 400 ±	2700
	PHA	256 ±	43
	PMA + CD28 mAb 9.3	48 000 ±	7500

[a]For the purposes of comparison, the proliferation induced by CD3sp and PMA + ionomycin can be expressed as a percentage of the maximal T cell proliferation induced in each experiment by PMA + CD28 mAb 9.3. Expressed in this manner CD3sp induces 60.3% and PMA + ionomycin induces 61.2% of the maximal proliferation induced by PMA + CD28 mAb 9.3. Differences in absolute levels of thymidine incorporation between the two experiments reflect differences in the relationship between the 8-h labeling period and the peak of cell proliferation during the third day of culture.
[b]'CD28' T cells were cultured at 1×10^5/well in RPMI + 5% FCS with CD3sp at 50 ng well, PHA at 3 μg/ml, PMA at 3ng ml, mAb 9.3 at 1 μg ml or ionomycin at 0.2 μg/ml.
[c]Thymidine incorporation (arithmetic mean ± SEM) was determined on day 3 of culture.

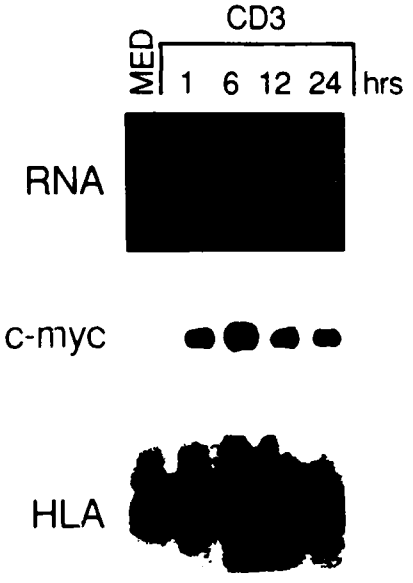

Fig. 1. c-*myc* mRNA levels following crosslinking of the TCR CD3 receptor complex on resting peripheral blood T cells. Resting peripheral blood T cells were stimulated by crosslinking the TCR CD3 receptor complex using a CD3-specific mAb G19-4. RNA was isolated from the cells prior to stimulation and at 1, 6, 12 and 24 h following activation. Total cellular RNA was isolated and equalized for rRNA as previously described (Thompson *et al.*, 1985). The equalization was confirmed by ethidium bromide staining of 10% aliquots of each RNA sample separated on non-denaturing 1% agarose gels (upper panel). Northern blots were prepared as described in Materials and methods and the filters were hybridized sequentially with radiolabeled cDNA probes for the c-*myc* and HLA-B7 (HLA) genes.

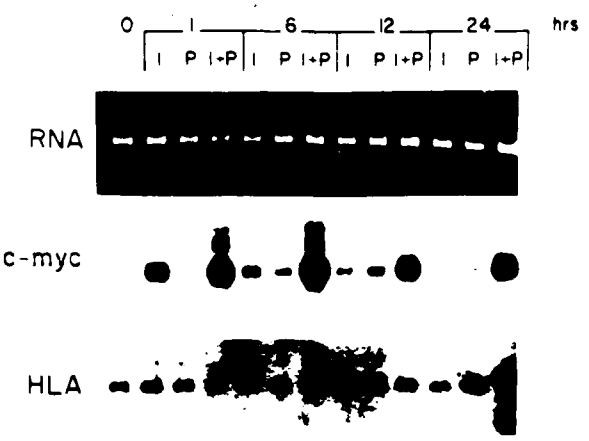

Fig. 2. Induction of c-*myc* gene expression by PMA and ionomycin. Purified resting T cells were incubated with ionomycin (I), PMA (P) or ionomycin + PMA (I + P) for 1, 6, 12 and 24 h. RNA was isolated and equalized as described in the legend to Figure 1. Upper panel represents ethidium bromide staining of the equalized RNA samples. Northern blots were sequentially hybridized to the radiolabeled c-*myc* and HLA-B7 (HLA) cDNA probes.

C (Truneh *et al.*, 1985; Ledbetter *et al.*, 1987). These two pathways can be stimulated independently *in vitro*. Cytoplasmic free calcium levels can be increased by the calcium ionophore, ionomycin. Membrane translocation and activation of protein kinase C can be induced by PMA. Neither agent alone is capable of inducing proliferation of resting peripheral blood T lymphocytes (Table I). However, the combination of PMA + ionomycin treatment leads to levels of proliferation equivalent to those observed following crosslinking of the TCR/CD3 complex. To determine which of these two pathways might be primarily responsible for the activation of c-*myc* following surface stimulation of the TCR/CD3 complex, we investigated c-*myc* mRNA levels following stimulation of resting peripheral blood T cells by ionomycin alone, PMA alone or the combination of PMA + ionomycin. RNA was isolated at 0, 1, 6, 12 and 24 h after stimulation of cells, and was analyzed for c-*myc* mRNA levels by Northern blot analysis (Figure 2).

Ionomycin treatment alone led to a 10- to 20-fold induction of c-*myc* mRNA levels within 1 h following stimulation. The ionomycin-induced expression of c-*myc* was transient, and by 24 h of stimulation ionomycin-treated cells showed no increase in c-*myc* mRNA levels above background. In contrast, when cells were stimulated with PMA, a slow steady rise in c-*myc* mRNA was noted over the first 12–24 hours of culture. The total magnitude of c-*myc* gene expression, however, was only 2–4 times greater than the c-*myc* mRNA levels of unstimulated peripheral blood T lymphocytes. When ionomycin and PMA were added together, c-*myc* mRNA levels increased within 1 h of stimulation, reached a transient peak in expression 6 h following stimulation and remained expressed at a high level throughout the proliferative response. The kinetics of the c-*myc* mRNA induction produced by treatment with ionomycin + PMA mimicked those exhibited by stimulation of peripheral blood T cells by crosslinking of the TCR CD3 receptor. The specificity of these changes in c-*myc* expression was demonstrated by hybridization of the same blot with a probe specific for an HLA class I gene. These data confirm previous observations suggesting that c-*myc* gene expression alone is insufficient to induce proliferation in normal cells (Coughlin *et al.*, 1985). Reconstitution of the pattern of c-*myc* gene expression following crosslinking of the TCR/CD3 complex requires the simultaneous stimulation of cells with both ionomycin and PMA.

Induction of c-myc gene expression by ionomycin and PMA differ in their requirements for new protein synthesis

Both the magnitude and kinetics of c-*myc* mRNA expression following PMA as compared to ionomycin stimulation suggested that activation of these two intracellular pathways might lead to increased c-*myc* gene expression by independent molecular mechanisms. To investigate the role of new protein synthesis in inducing c-*myc* gene expression as the result of stimulation by these pathways, quiescent cells were stimulated with ionomycin or PMA in the presence or absence of cycloheximide at concentrations sufficient to suppress 95% of protein synthesis (Figure 3). When resting peripheral blood T cells were stimulated with cycloheximide alone for 2 h, a 2- to 4-fold increase in c-*myc* mRNA levels was observed. This is consistent with the observation of others that cyclohexmide is capable of inducing c-*myc* mRNA levels by increasing mRNA stability in the cytoplasm (Dani *et al.*, 1984). When cells were treated with ionomycin in the presence of cycloheximide, a 'superinduction' of c-*myc* mRNA levels was observed. In contrast, when cells were stimulated with PMA in the presence of cycloheximide, the induction of c-*myc* mRNA levels by PMA was completely abolished. c-*myc* gene expression in the presence of PMA + cycloheximide was entirely accounted for by the induction mediated by cycloheximide alone. These results suggest that the PMA-mediated induction of c-*myc* mRNA levels requires new protein synthesis. To confirm this observation, cells were pretreated with PMA for 12 h to allow new protein synthesis to occur, and then cycloheximide was added to the media. Under this set of conditions, cycloheximide leads to a further enhancement of the levels of c-*myc* mRNA induced by PMA. The combination of PMA and ionomycin can also be shown to be superinduced by cycloheximide when cells were first pretreated with PMA for 12 h and then stimulated with ionomycin in the presence or absence of cycloheximide for 2 h. The combination of PMA and ionomycin not only leads to increases in c-*myc* transcripts initiated from the major promoters, P1 and P2, but also to increases in the 3.1-kb c-*myc* transcripts initiated from the P0 promoter region (see Figure 5D for map). A similar induction of the 3.1-kb c-*myc* transcript was also seen in CD3sp-stimulated cells (Figure 1) and in cells treated concomitantly with PMA and ionomycin (Figure 2).

Cycloheximide induces c-myc gene expression in quiescent T cells by increasing mRNA stability

The preceding results suggest that c-*myc* gene expression can be independently induced by three distinct molecular mechanisms. To confirm this we analyzed the role of various transcriptional and post-transcriptional mechanisms in inducing c-*myc* gene expression in response to cycloheximide, PMA and ionomycin. The role of alterations in mRNA stability in mediating the induction of c-*myc* mRNA by PMA, ionomycin and cycloheximide was investigated by inducing c-*myc* mRNA expression to optimal levels via all

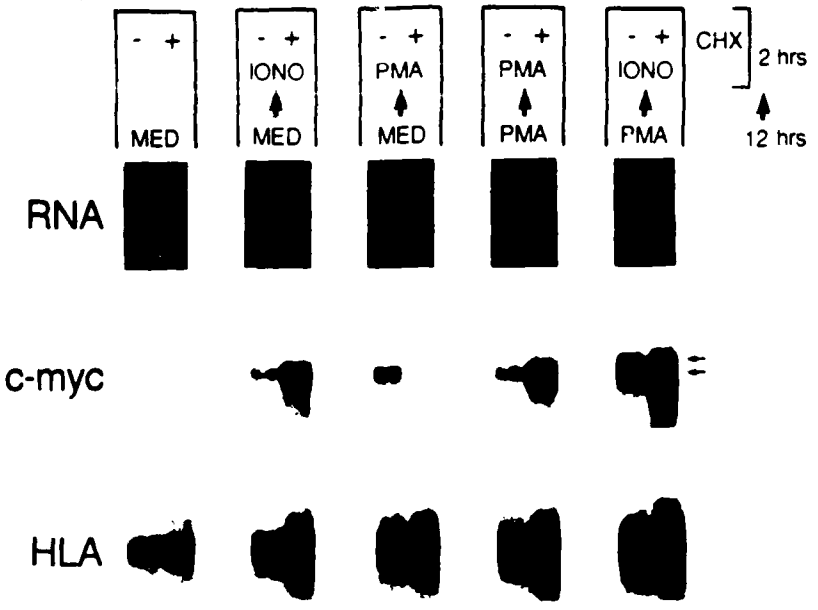

Fig. 3. The effects of the protein synthesis inhibitor cycloheximide on PMA- and ionomycin-induced c-*myc* gene expression. Purified resting T cells were treated with media alone, ionomycin or PMA for 2 h in the presence or absence of cycloheximide. In addition, resting peripheral blood T cells pretreated with PMA for 12 h were further stimulated with PMA or ionomycin in the presence or absence of cycloheximide for an additional 2 h. RNA was isolated and equalized as described in legend to Figure 1. Filters were sequentially hybridized to the radiolabeled c-*myc* and HLA-B7 (HLA) cDNA probes. The upper arrow on the right-hand side of the c-*myc* panels indicates the positions of the 3.1-kb P0-initiated message present in the PMA pretreated cells stimulated subsequently with ionomycin in the presence or absence of cycloheximide. The lower arrow depicts the migration of the 2.2/2.4-kb c-*myc* transcripts initiated from the major promoters. The identity of the 3.1-kb transcript in the ionomycin + PMA treated cells was confirmed using the *AccI–PstI* fragment described in Materials and methods, previously shown to be specific for the P0-initiated transcripts (Bentley and Groudine, 1986b).

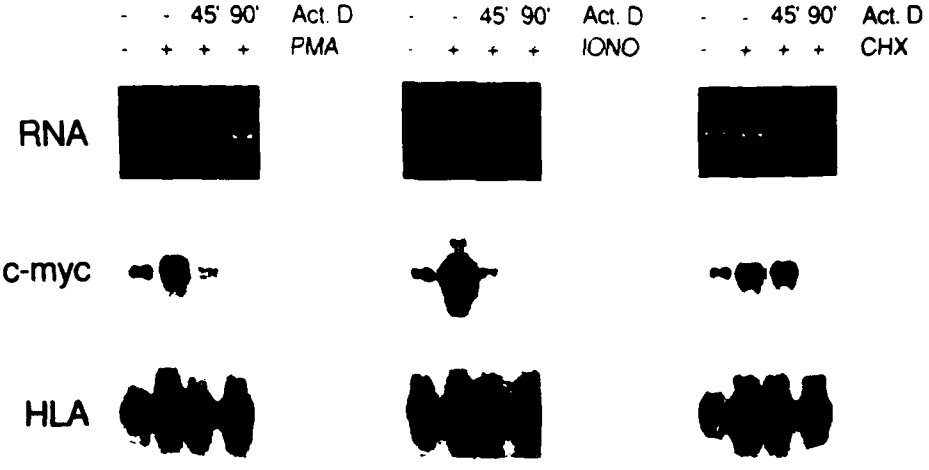

Fig. 4. The half-life of c-*myc* mRNA in T cells treated with either PMA, ionomycin or cycloheximide. Purified resting T cells were stimulated with PMA for 12 h, ionomycin for 2 h or cycloheximide for 3 h. At the end of the stimulation period actinomycin D was added to the cultures. RNA was isolated from the cells at the beginning and end of the stimulation period with each agent and after 45 and 90 min following the addition of actinomycin D. The upper panels show the ethidium bromide staining of equalized RNA samples. Northern blots were sequentially hybridized with radiolabeled c-*myc* and HLA-B7 (HLA) cDNA probes.

three pathways, and then analyzing the rate of c-*myc* mRNA degradation following the addition of actinomycin D (Figure 4). The half-life of c-*myc* mRNA induced by ionomycin or PMA is ~15 min. A similar half-life was determined for the low levels of c-*myc* mRNA expressed in unstimulated T cell controls (data not shown). In contrast, the c-*myc* mRNA half-life was prolonged to >1 h following cycloheximide treatment of resting cells. This is consistent with the observations that cycloheximide induces c-*myc* mRNA level by increasing mRNA stability (Dani *et al.*, 1984; Thompson *et al.*, 1986). These results also suggested that both PMA and ionomycin regulate c-*myc* mRNA expression by affecting the transcription rate and/or the processing of the primary c-*myc* transcripts.

Ionomycin and PMA induce c-myc expression by independent mechanisms

To investigate the role of alterations in transcriptional regulation in the induction of c-*myc* gene expression by PMA and/or ionomycin, run-on transcription assays were performed. In these experiments, nuclei were isolated from resting peripheral T lymphocytes following stimulation with media alone, PMA, ionomycin, PMA + ionomycin, or cycloheximide. Nuclei were then incubated with ^{32}P-radiolabeled UTP under conditions that allowed for elongation of transcripts initiated by polymerases already bound to DNA. Radiolabeled nascent RNA transcripts were isolated from the reaction mix and hybridized to filters containing single-stranded M13 probes specific for exon 1 and exon 2 in both the sense and anti-sense orientation, and a double-stranded probe for the P0 promoter region of the c-*myc* gene. A full-length glyceraldehyde-3-phosphate dehydrogenase (GPD) cDNA was used as a control template. Resting peripheral blood T cells display significant hybridization to the sense strand of exon 1, demonstrating that even in the resting state the c-*myc* major promoters, P1 and P2, are being utilized by polymerases (Figure 5A). However, significantly less hybridization was observed using an exon 2 template of comparable size. Comparison of the band intensities between the exon 1-sense strand and the exon 2-sense strand suggests that there is a 5- to 10-fold block in transcriptional elongation between exon 1 and exon 2 in resting T cells. Previously reported studies have suggested that this block to elongation occurs at the exon 1/intron 1 boundary (Bentley and Groudine, 1986a, 1988; Nepveu and Marcu, 1986; Eick and Bornkamm, 1986), and we have performed no further studies to define this region. A low level of hybridization to the upstream P0 promoter region was also observed. Similar results were obtained when resting T cells were incubated with cycloheximide for 2 h and c-*myc* transcription evaluated by the run-on transcription assay. This observation is consistent with our finding that cycloheximide enhances c-*myc* mRNA levels by increasing the c-*myc* mRNA half-life.

When resting peripheral blood T lymphocytes were incubated for 14 h with PMA, a 4- to 5-fold decrease in the polymerase density on the sense strand of exon 1 was observed (Figure 5A). In contrast, only slight decreases in the hybridization to the exon 2-sense strand and to the P0 and GPD plasmids were observed. Despite the overall decrease in transcription, there is a reproducible decrease in the ratio of exon 1 to exon 2 transcription in PMA-treated cells as compared to untreated resting T cells. This reduction in the block to transcriptional elongation appears to require new protein synthesis. Quiescent cells treated simultaneously with PMA and cycloheximide failed to exhibit any alterations in the hybridization intensities of exon 1 and exon 2 when compared with untreated cells (data not shown). In addition, when PMA pretreated cells were incubated with cycloheximide the ratio of exon 1 to exon 2 transcription increased to the level seen in untreated quiescent cells within 2 h of cycloheximide addition. While these results suggest there has been a reduction in the block to transcriptional elongation within the c-*myc* gene as a result of PMA treatment, the observed differences in c-*myc* transcription do not account for the increase in c-*myc* mRNA that occurs following PMA stimulation of resting T cells. This would suggest that the ability of PMA to induce c-*myc* expression may either be

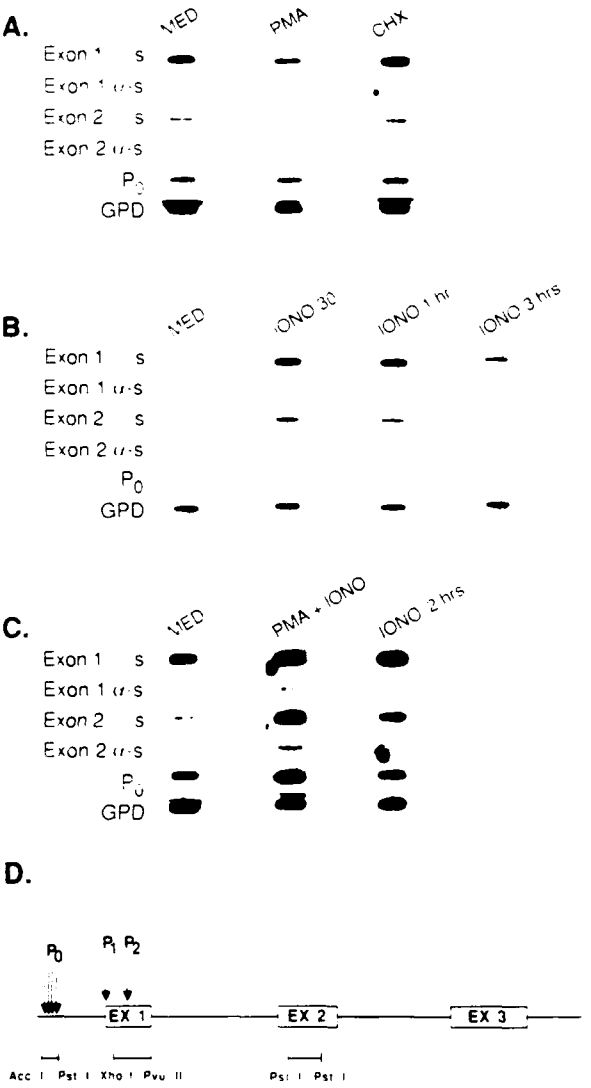

Fig. 5. c-*myc* transcription in peripheral blood T cells either resting, or treated with PMA, ionomycin, PMA + ionomycin or cycloheximide. (**A**) run-on transcription assays were performed on resting peripheral blood T cells incubated for 14 h in the presence of media alone, PMA or 12 h of media followed by the addition of cycloheximide for 2 h of culture. Nuclei were isolated and run-on transcription assays were performed as described in Materials and methods. Run-on transcription products were hybridized to filters containing genomic c-*myc* probes specific for exon 1-sense, exon 1-antisense, exon 2-sense, exon 2-antisense, a plasmid containing the *Acc*I – *Pst*I fragment specific for P0 transcription, and a plasmid containing the GPD gene. Run-on transcription assays were also performed in the presence of 2 μM α-amanitin, which completely abolished hybridization to the c-*myc*-specific fragments (data not shown). (**B**) Resting peripheral blood T cells were stimulated with ionomycin for 30 min, 1 h and 3 h and nuclei isolated from the cells prior to stimulation and following ionomycin treatment for 30 min, 1 h and 3 h. Run-on transcription products were hybridized to blots prepared as described in (A). (**C**) Cells were incubated with media alone for 14 h, PMA alone for 12 h followed for an additional 2 h by the addition of ionomycin to the culture. A third aliquot was incubated in media alone for 12 h and ionomycin added for an additional 2 h. Run-on transcription productions were hybridized to nitrocellulose filters as described above and the resulting autoradiogram depicted. (**D**) Genomic map of the c-*myc* gene depicting the positions of the three exons, the major promoter P1 and P2, and the P0 promoter region. Positions of the probes used in the run-off transcription assays are shown.

multifactorial or resides at the level of the efficiency with which the primary transcript is processed.

In contrast to these results, when resting peripheral blood cells are stimulated with ionomycin a >10-fold increase in the hybridization to both exon 1 and exon 2 is seen within 30 min following stimulation of the cells (Figure 5B). The increase in transcription can entirely account for the increase in c-*myc* mRNA that is observed following ionomycin treatment of resting T cells. However, despite this increase in overall transcription in the locus, a significant block to transcriptional elongation between exon 1 and exon 2 is still present. Addition of cycloheximide with ionomycin had no effect on the transcriptional effects of ionomycin (data not shown).

We next examined the transcription of c-*myc* mRNA in cells stimulated with both PMA and ionomycin. In cells pretreated with PMA for 12 h and subsequently stimulated with ionomycin for 2 h, the transcription rate of exon 1 as assayed by run-on transcription appears to be increased to a level similar to that induced by treatment with ionomycin alone (Figure 5C). However, in contrast to ionomycin treatment, the hybridization to exon 2 in PMA + ionomycin treated cells is equivalent of that of exon 1. These observations suggest that following ionomycin activation of PMA-pretreated cells there is both an increase in transcription initiation within exon 1 and a reduction of the block to transcription elongation within the gene. Addition of cycloheximide to these cells did not affect the increase in transcriptional initiation but resulted in a return of the block to transcriptional elongation within 2 h of addition. The combination of PMA + ionomycin was also found to increase the rate of transcription within the P0 promoter region of the gene. This is consistent with our observation that ionomycin + PMA leads to a significant induction of P0-initiated full-length c-*myc* transcripts.

Discussion

Our data suggest that there are at least three independent mechanisms that account for the low level of c-*myc* gene expression in normal resting peripheral blood T cells. These include a low constitutive level of promoter utilization at the 5′ end of the gene, a block to transcriptional elongation within the gene and a rapid rate of c-*myc* mRNA degradation in the cytoplasm. Surface activation events mediated through the TCR/CD3 receptor can initiate both the expression of c-*myc* mRNA and cellular proliferation in polyclonal populations of normal peripheral blood T lymphocytes. Previous studies have shown that the TCR/CD3 receptor mediates its effects through two intracellular pathways—activation of protein kinase C and increased intracellular calcium. Our data support this observation by demonstrating that the combination of the activation of protein kinase C by PMA and the increase of intracellular calcium levels by ionomycin leads to c-*myc* expression and cell proliferation that is similar in level and kinetics to that initiated by surface crosslinking of the TCR/CD3 receptor. Together, PMA and ionomycin induce c-*myc* transcription by increasing promoter utilization and by reducing the block to transcriptional elongation within the gene.

The major quantitative mechanism leading to c-*myc* mRNA expression in resting T cells is mediated by increased transcriptional initiation. This mechanism can be activated by elevation of intracellular ionized calcium through treatment of resting T cells with ionomycin. Elevations in intracellular calcium have been reported in the transcriptional activation of several other genes (Lin *et al.*, 1986; Resendez *et al.*, 1986). Our data show that the induction of c-*myc* gene expression by the calcium ionophore ionomycin is independent of new protein synthesis. In contrast, the calcium ionophore-mediated transcriptional induction of the glucose-related genes P3C5 and P4A3 has been shown to require new protein synthesis (Resendez *et al.*, 1986). Together these results suggest that elevation of the intracellular calcium level can activate several molecular mechanisms that may affect the transcription of individual genes.

A reduction in the block to transcriptional elongation can also contribute to increase c-*myc* expression following activation of quiescent T cells. Reduction in the block to transcriptional elongation was most clearly seen in cells pretreated with PMA and then stimulated with ionomycin. A reduction in the block to transcriptional elongation was also observed in cells treated with PMA alone. In both instances treatment with the protein synthesis inhibitor cycloheximide led to full recovery of the block to transcriptional elongation within 2 h. These results suggest the PMA-induced pathway complements the ionomycin-mediated transcriptional induction of c-*myc* by leading indirectly to a decrease in the block to transcriptional elongation. Alternatively, signals provided by both PMA and ionomycin may normally be required for optimal reduction of the block to transcriptional elongation. Such an interaction between ionomycin and PMA was demonstrated by the ability of these two agents to increase synergistically transcription 5′ of the major promoters. This increased transcription results in the induction of the previously reported 3.1-kb P0-initiated c-*myc* transcripts (Bentley and Groudine, 1986b). Our difficulty in determining a single molecular mechanism by which PMA alone leads to c-*myc* expression is consistent with previous reports that PMA induction contributes to gene expression via a number of distinct molecular mechanisms (Chiu *et al.*, 1987).

The importance of cytoplasmic degradation of messenger RNA in regulating the final level of c-*myc* was demonstrated by treatment of resting peripheral blood T lymphocytes with cycloheximide. Such treatment leads to a significant increase in c-*myc* mRNA levels. This result demonstrates that there is enough full-length transcription of the c-*myc* gene even in the resting state to allow for cytoplasmic accumulation of the mRNA in the absence of selective c-*myc* mRNA degradation. Thus, rapid degradation of c-*myc* mRNA is an important mechanism by which quiescent T cells down-regulate their expression of c-*myc*. Several groups have suggested that the ability to turn off c-*myc* gene expression is required for cells to enter a resting state (Dean *et al.*, 1986; Lomo *et al.*, 1987; Freytag, 1988). So far, we have not been able to demonstrate a physiologic mechanism that regulates variations in the c-*myc* mRNA half-life in T cells, as has previously been demonstrated to occur in normal fibroblasts (Blanchard *et al.*, 1985). However, a number of alternative pathways that contribute to T cell activation have recently been described involving lymphokines and cell surface receptors distinct from the TCR/CD3 antigen receptor complex (Reed *et al.*, 1985; June *et al.*, 1987; Weiss and Imboden, 1987). Further study of T cell proliferation involving these additional surface activation events may well

reveal that alteration in mRNA half-life is an important mechanism by which T cells augment c-*myc* mRNA levels during cellular proliferation.

In summary, our data suggest that there are three important levels of regulation of c-*myc* mRNA in peripheral blood T cells. Upstream promoter utilization within the c-*myc* proto-oncogene appears to be inducible by increases in intracellular calcium. The block to transcriptional elongation within the c-*myc* gene appears to be reduced as a result of new protein synthesis induced by either PMA or PMA + ionomycin treatment of quiescent cells. Both of these transcriptional effects are mediated through intracellular pathways that are induced by activation of the TCR/CD3 receptor complex. Thus, both promoter utilization and transcriptional attenuation are important physiologic mechanisms by which c-*myc* mRNA levels are regulated during the activation of normal peripheral blood T cells. In addition, the rapid degradation of c-*myc* mRNA in the cytoplasm appears to contribute to the low level of c-*myc* mRNA expression present in quiescent peripheral blood T lymphocytes.

Materials and methods

Cells

Resting human peripheral blood T lymphocytes were isolated by negative selection according to a previously published protocol (June *et al.*, 1987). In brief, peripheral blood lymphocytes were obtained by leukapheresis of healthy donors of age 21–31 years. Residual red blood cells were removed by density gradient centrifugation. The peripheral blood lymphocytes were then incubated at 4°C on a rotator with saturating amounts of monoclonal antibodies (mAb) 60.1 (anti-CD11), 1F5 (anti-CD20), FC-2 (anti-CD16) and 63D3 (anti-CD14) for 20 min. This mixture of antibodies coated all B cells, monocytes, large granular lymphocytes and CD11-bearing T cells with mouse immunoglobulin. The cells were washed three times to remove unbound antibodies and then incubated with goat anti-mouse immunoglobulin-coated magnetic particles. Antibody-coated cells that were then bound to beads were removed by magnetic separation. Typically, $5-10 \times 10^8$ cells were recovered. Cell purification was routinely monitored by flow cytometry and histochemistry. Monocytes, B cells and large granular lymphocytes were not detectable by immunofluorescence. Residual monocytes were quantitated by staining with non-specific esterase and were <0.1% in all cell populations. Viability was >99% as measured by trypan blue exclusion. The resulting cell populations were >99% CD2 positive as determined by flow cytometry.

Proliferation assays

Cells were cultured in quadruplicate samples in flat-bottomed 96-well microtiter plates at 1×10^5 cells/well in RPMI-1640 media containing 5% heat-inactivated fetal calf serum. Cell proliferation was measured in a liquid scintillation counter after pulsing cells for the last 8 h of 3-day cultures with 1 μCi/well of [^{3}H]thymidine. Stimulation of the cells was performed with either media alone as described above, or with anti-CD3 mAb G19-4, which was produced and purified as described previously (June *et al.*, 1987). To induce surface crosslinking of CD3, necessary for CD3-mediated T cell proliferation, mAb G19-4 was adsorbed to the surface of plastic tissue culture plates. Monoclonal antibody 9.3 was added, as previously described (June *et al.*, 1987), at an optimal concentration of 1 ng/ml. Phytohemagglutinin (PHA) was used at 3 μg/ml, a concentration found in preliminary experiments to result in optimal proliferation of unpurified peripheral blood lymphocytes. Ionomycin was added to cultures to give a level of 400 ng/10^6 cells. PMA was used at a concentration of 3 ng/ml. These doses of ionomycin and PMA were found to result in optimal proliferation of purified T cells.

Northern blot analyses

Purified T cells were cultured at 2×10^6/ml in complete media with either PMA at 3 ng/ml, ionomycin at 800 ng/ml, or a combination of both. In some experiments the cells were treated with actinomycin D at 5 μg/ml to inhibit RNA synthesis or cycloheximide at 10 μg/ml to suppress protein synthesis. Cells were harvested by centrifugation and total RNA extracted using guanidinium isothiocyanate (Chirgwin *et al.*, 1979). The resulting RNA samples were equalized for rRNA and the equalization confirmed by ethidium bromide staining of the RNA samples separated on non-denaturing 1% agarose gels (Thompson *et al.*, 1985). The equalized RNA samples (5–10 μg) were separated on 1% agarose–formaldehyde gels and transferred to nitrocellulose. Membranes were baked under vacuum for 2 h and then hybridized at 42°C in a solution containing 50% formamide, 5 × SSC, 1 × Denhardt's solution, 25 mM sodium phosphate (pH 6.5) and 250 μg/ml of Torula RNA. Hybridizations were carried out for 16–20 h under identical conditions with the addition of 10% dextran sulfate and 1×10^6 c.p.m./ml of the DNA probe. DNA probes were labeled by nick translation to a specific activity of $3-9 \times 10^8$ c.p.m./μg. Following hybridization, membranes were washed briefly at room temperature in 2 × SSC, 0.1% SDS, and then for 2 × 30 min at 56°C in 0.1 × SSC, 0.1% SDS. The membranes were then air dried and exposed to X-ray film (Kodak XAR-5) for 2–48 hours at −70°C using an intensifying screen. Band intensities were compared using densitometry, as previously described (Thompson *et al.*, 1986).

DNA probes

Three c-*myc* gene specific probes were used for the analysis of c-*myc* mRNA levels on Northern blots. A 1-kb *Cla*I–*Eco*RI fragment was isolated from a human c-*myc* cDNA and a 200-bp *Acc*I–*Pst*I fragment that includes the P0 promoter region, both previously reported and provided by D. Bentley (Bentley and Groudine, 1986b). Run-on transcription assays were performed using an exon-1 specific *Xho*I–*Pvu*II 443-bp fragment cloned into M13 in both the sense and anti-sense orientations, and a 414-bp *Pst*I fragment from the second exon of the gene cloned in the sense and anti-sense orientation of M13, as previously described and generously provided by D. Bentley and M. Groudine (Bentley and Groudine, 1986a). P0 transcription was assayed using the *Acc*I–*Pst*I fragment cloned into pSP65 as described above. The human leukocyte antigen (HLA-B7) probe was a 1.4-kb *Pst*I fragment isolated from pHLA-B7 (Sood *et al.*, 1981). The glyceraldehyde-3-phosphate dehydrogenase (GPD) cDNA probe has been previously described (Dugaiczyk *et al.*, 1983). Inserts (50–100 ng) of the above-mentioned plasmids, obtained following digestion with appropriate restriction endonucleases and separation on low-melting-point agarose, were labeled by nick translation to a specific activity of $3-9 \times 10^8$ c.p.m./μg. Labeled probes were added to a final concentration of 10^6 c.p.m./ml hybridization mix.

Run-on transcription assays

Nuclei were prepared as previously described (Groudine *et al.*, 1981) and resuspended in 50 mM Tris–HCl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA. Nuclear run-on transcription assays were performed as described by Groudine *et al.* (1981) and modified elsewhere using ^{32}P-labeled UTP (Linial *et al.*, 1985). Generally, 10^7 c.p.m./5×10^7 cells were obtained. An equal number of counts per minute were hybridized to slot blots containing 5 μg of double-stranded plasmids or 0.5 μg of M13 clones. The plasmids used are described above. Hybridization and washing conditions have been described elsewhere. Slot blots were prepared as previously described. Each experiment was performed several times to confirm the reproducibility of our results. In some experiments 2 μM α-amanitin was added to nuclei prior to the start of the run-on transcription assay to inhibit polymerase II activity. Under the conditions of our assay, this dose of α-amanitin was sufficient to inhibit all observed transcription within the c-*myc* locus but left polymerase I and polymerase III activity unaffected as assayed by hybridization to 28S ribosomal and 5S ribosomal clones respectively (J. Battey and C. Thompson, unpublished data). Bentley and Groudine (1988) have similarly shown that both the transcriptional initiation and the block to transcriptional elongation within the c-*myc* gene are the result of polymerase II transcription.

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